

## **Tree-dimensional Organization of Thylakoid System in Granal Chloroplasts and its Functional Role**

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**Abstract.** In the chloroplasts of mesophyll cells of C<sub>3</sub> and C<sub>4</sub> plants the thylakoid membranes shows multiple helices of stroma lamellae around the cylindrical grana. Although this spatial relationship has been known for more than two decades, most textbooks and research papers continue to contain erroneous 3D models and simplified schemes. These reviews presents a detailed computer model, based on electron microscopic observations and explain the ontogenetic formation and functional importance of this unique structure.

### **Development of 3-dimenzional view**

The electron microscopic investigations of thin section of chloroplast showed numerous flattened membrane sacs, called thylakoid. Thylakoid membranes are the internal membranes of chloroplasts, which accommodate all light-harvesting and energy-transducing functions. They are embedded in an aqueous matrix, called the stroma, which is surrounded by double envelope membrane (Fig 1). The thylakoids fell into two general size ranges. The smaller sizes around 0.5 µm in diameter are appressed and form grana stacks. The larger sizes, called stroma thylakoids, are continuation of every second or third grana and interconnect the grana stacks. From this view was constructed the first simple model by Menke (1960). Weier *et al.*, constructed a tubular model in 1961, which shows an anastomosing system of membranous channels. This concept was an artifact caused by KMnO<sub>4</sub> instead of glutaraldehyde fixation (Falk and Sitte, 1963).

The further detailed studies on the sections revealed, that in some cases the stroma thylakoid is not continued from one granum disc but interconnect

two of them. From this arrangement it was concluded, that the stroma thylakoids could intersect the granum at an angle. In the three-dimensional interpretation by Heslop-Harrison (1962) showed, that one stroma lamella interconnects all the grana compartments. The consequence of this arrangement was that all the interthylakoidal space (loculus or lumen) might be a continuum in the lamellar system. This idea was further persuaded by Wehrmeyer (1964), who proposed that a single stroma lamella could exist in a spiral or helical arrangement around the granum being connected a few individual granum compartment. This general idea was elaborated by Paolillo (1970), who proposed that more than one stroma lamella could be helically attached and confirmed with the aid of a complete series of sections (Mustárdy and Brangeon 1978; Brangeon and Mustárdy 1979). The helical organization of the stroma lamellae around the grana can also be seen in other, more conventional (i.e. non-serial-section) electron micrographs. For instance, in thin sections the helices become evident when the section plane crosses the marginal region of the granum (Fig. 3a). In this relatively rare case, the thin section exposes the stroma membranes in their tilt positions and the granum stacks remain masked, or not included in the section.

The helical arrangement of the stroma thylakoids can also be seen on freeze etching replicas (Staehelin, 1986) (Fig. 3b) and it is most clear in scanning electron micrographs of isolated grana obtained by sonication and differential centrifugation (Mustárdy and Jánossy, 1979) (Fig. 3c). This figure shows that a helical strip of the stroma membranes covers the granum 'pillar'.

Based on these electronmicroscopical observations a detailed computer model was constructed, showing the striking 3D structure with the stroma membrane helically wound around the granum (Mustárdy and Garab, 2003) (Fig. 4).

These helical granum-stroma assemblies are fused together as it can be seen on the serial section electron micrographs, i.e. the cylindrical granum of stacked membranes surrounded by multiple helices of the stroma thylakoids, are interconnected by fusion at the 'outer edges' of stroma membranes (Fig. 5). Evidently, this can be extended to the entire chloroplast, which lends a substantial stability to the thylakoid membrane system. It is shown by sequential thin sections that – as a consequence of the helical organization – the stroma membranes originating from one level of the multilamellar granum run to different levels on the 'opposite side' (i.e. on the interconnected granum) (Fig. 5a-c). The electron micrographs also show that the width of the pillars, i.e. the relative lengths of the granum membranes with respect to the stroma thylakoids, can vary inside a single granum. This probably reflects local variations in the composition during the build-up of the granum structure. Clearly, transient de-stacking of membranes in the marginal sections can also

induce such variations, and thus the membrane system must therefore be considered to exhibit substantial structural flexibility.

### **Functions based on ultrastructure**

More than 30 years ago Boardman and Anderson (1964) have revealed that the differentiation of thylakoids into granum and stroma membrane regions reflects a compositional and functional difference. The two photosystems are spatially separated: photosystem II (PSII) and its main chlorophyll a/b light-harvesting complex, LHCII, are found predominantly in the stacked membranes; this region is largely deficient in photosystem I (PSI) and LHCI, which are enriched in the stroma membranes (Andersson and Anderson, 1980). Separation of the two pigment systems is probably important in preventing unregulated excitation energy flow between the two photosystems (Anderson and Andersson, 1988; Trissl and Wilhelm 1993). Without this, PSI, which is much faster than PSII, would disturb the balance of the energy distribution between the two photosystems. It is known that LHCII has large self-aggregation ability. Indeed, LHCII has been shown to stabilize the granum ultrastructure, and to participate in the cation-mediated stacking of the membranes (Arntzen, 1978; Duniec et al., 1981; Barber, 1982). The abundance of LHCII in the granum suggests that these antenna complexes also play a structural role to separate the two photosystems. These light-harvesting complexes have also been shown to be involved, via electrostatic and osmotic forces, in the lateral organization of the membranes (Garab et al. 1991). Separation of the two photosystems (the LHCII-PSII and LHCI-PSI supercomplexes) between stacked and unstacked regions evidently requires a lateral force, such as that which governs the formation of LHCII-containing macrodomains (Garab and Mustárdy, 1999). The fact that in all LHCII-containing chloroplasts the two photosystems are segregated between the granum and stroma membranes suggests that LHCII may be involved not only in stacking but also in the lateral separation of PSII and PSI.

The helical arrangement of the stroma thylakoids around the cylindrical granum stacks has several functions. The continuity of the membrane is obviously warranted within the same granum-stroma assembly. The fusion of two (or more) stroma membranes at the edges ensures the continuity between the two types of membrane for the entire chloroplast. The continuum of the membrane system is evidently important for the diffusion of mobile components between the two regions (like plastocyanin). The 3D organization of the membrane may have special significance in the PSII repair cycle, during which the damaged particles are transferred from the granum to the stroma membranes where they are repaired and reassembled and then transferred back to the stacked region (cf. Melis 1991; Russel *et al.*

1995). It is tempting to speculate that the repair, which is a complex process, can be performed only at certain sites where both the key enzymes and the ribosomes are available, e.g. on the 'top' of the stroma membranes which is fully exposed to the stroma liquid. The spiral organization of these membranes and the multiple connections to the granum can facilitate the diffusion of the components from these special sites to any target. A similar mechanism may apply virtually to all components of the granum membranes, which are inserted in the stroma membranes and then 'slide' to the stacked region.

At the same time the openings on the 'sacs' ensure ionic conductance in a lateral direction between the inner aqueous phases of granum and stroma thylakoids. Further, stroma thylakoids by spiralling up and around the stacks ensure that all inner compartments of a granum are electrically connected. Finally, fusions of adjacent stroma thylakoids wound around neighboring stacks interconnect adjacent grana (cf. Mustárdy 1996). By this means the inner aqueous phases of all thylakoids in the chloroplast become electrically connected to each other. The electrochemical gradients are rapidly 'delocalised' for the entire thylakoid system, and thus the transmembrane pH and electric potential gradients become essentially uniform (Junge 1977; Mitchell 1977).

### Ontogenetic assembly of thylakoids

The spatial relationship of grana and stroma thylakoids during chloroplast differentiation can be most successfully studied by serial section analysis at different levels of ontogenesis. This approach was taken with *Lolium multiflorum* seedlings (Mustárdy and Brangeon, 1978; Brangeon and Mustárdy, 1979) in which four developmental stages of leaves were selected to follow the ontogenetic assembly of thylakoids by analysis and reconstitution of serial sections of developing chloroplasts.

In meristematic tissues the intrachloroplastic lamellae show a few pro-thylakoids of varying length distributed apparently at random within the proplastids (Fig. 6a). However, views of reconstituted serial sections reveal a single lamellar entity rather than separate and independent units. Thylakoids can be traced to a „parent entity” which has apparently split and given rise to several branches of different length. In some cases, the pro-thylakoid form is flattened and funlike, although apparently flexible and capable of changing orientation (Fig. 6b). In crossing this lamellar sheet, sharp cross-sectional views deteriorate into fuzzy tangential views. Some of the pro-thylakoids show a balloon-or barrel-like form with several marginal splits in the reconstituted serial sections (Fig. 6c). In cross section, these figures appear as roughly circular thylakoids with interruptions and/or as short separate lamellae, depending upon the section plane. A few rudimentary double

stacks are present at this stage. Within one proplastid, bubble-like invaginations of the inner membrane of the envelope are evident (see inset Fig. 6a) but the large, flattened lamellae are never connected to the inner envelope. Although, the possibility that these vesicles elaborate into thylakoid sheets was proposed by several authors (Hodge *et. al.*, 1956; Mühlethaler and Frey-Wyssling, 1959; Bradbeer *et. al.*, 1974), it has still not been proven.

The developing chloroplasts increase in size and become flattened. In the newly formed thylakoids most of the chlorophyll is present in the reaction centers. The light-harvesting complex, which facilitates stacking of membranes (Mullet and Arntzen, 1980), becomes associated only later with the reaction centers.

In this stage of chloroplast development the newly synthesized thylakoids are disposed parallel to one another and to the equatorial plane of chloroplasts (Fig. 7a). In the partial serial section the aligned thylakoids are revealed as individual broad sheets often traversing the width of the plastid (Fig. 7b). However, when the sections cross in a right angle, lamellar connections are encountered at the plastid ends (Fig. 7a arrow-heads). During this differentiation step there is an occurrence of multiple perforations dispersed throughout the lamellar sheets. This phenomenon has been sited in a variety of membrane systems, particularly in a rapidly expanding membrane (Dalton and Hagueneau, 1968). The interruptions give a beaded appearance to the thylakoids in the cross-section (Fig. 7a inset) but in the reconstituted view these are revealed as slits or tears (Fig. 7c). In this developmental stage several double or triple stacks can be observed which overlap on the top of and beneath of the lamellar sheet (Fig. 7a, arrows). The three-dimensional reconstitutions show discs of varying diameter in the course of expansion and reveal an overgrowing phenomenon (simultaneous grow and appress). These reconstitutions sometimes disclose remnants of slits alongside the overlaps which suggests that the perforations may be the sites of grana initiation. It is very likely that the edges of the lamellae are the growth points, while at the periphery of the holes, the overlapping growth pattern above and below the single lamellar sheet gives rise to stacking.

Very probable, that the appearance of light-harvesting complex at the growth points associated with PSII by its high self-aggregation capability initiates the granum formation. The lateral-aggregation of PSII particles via adhesion of peripheral light-harvesting complexes can lead to the formation of nearly homogenous region which are enriched in PSII and exclude PSI to a large extent. These so-called „macrodomains” through their head-to-head aggregations ensure the granal stacking (Garab *et. al.*, 1991; Barzda *et. al.*, 1994) and cause the separation of the two photosystems.

The parent lamellar sheet, interrupted in the vicinity of each nascent granum, constitutes a fret attachment at different levels to the stack. This arrangement maintains a continuity and consequently connects all grana that have been initiated on the same sheet. The „insertion” of the stacked discs into a lamellar sheet introduces a twist in the lamellae surrounding the developing granum, an orientation that would be the primary helix around the granum. Three-dimensional reconstitution of developing grana often reveal incomplete disc at the end of the stacks that have only a single fret connection. This shows that the granum can be increased by „overtopping” of the overgrown fret (Fig. 8b).

As the number of granum compartments increases, the fretwork is built into multiple layers. There are two types of configurations contributing to the fretwork increase. Firstly, the primary helix divide and directly give rise to a secondary helix (Fig. 8c). Secondly, numerous thylakoid „splittings” can be reconstituted during this stage (Fig. 8d). After ramifications the cleaved thylakoids extend and link into the existing helical system of neighboring granum or give an additional helix to the stack. This can happen not only in the same lamellar sheet but the growing thylakoid can also extend towards the lower (or upper) plane (Fig. 8a, arrows). From this developmental stage the parallel „parent” lamellae have been replaced by a network which interconnects at different levels.

These multiple mechanisms – lamellar expansion, perforation, overgrowth; thylakoid splitting, branching, and bridging *via* fusion – guide the morphogenesis of the intrachloroplastic network formation and lead to the mature chloroplast.

### Obsolete models still in use

The first model, proposed by Wilhelm Menke still used in some books and homepages, postulates that every second granum thylakoid is continued in sheets that intersect several grana (Fig. 2a). This oversimplified scheme does not satisfy the requirement of membrane continuity, and is inconsistent with the single intrathylakoidal (lumenal) space. This model was modified by Lexa and Crang (1997) (Virtual Cell

<http://www.life.uiuc.edu/plantbio/cell/>).

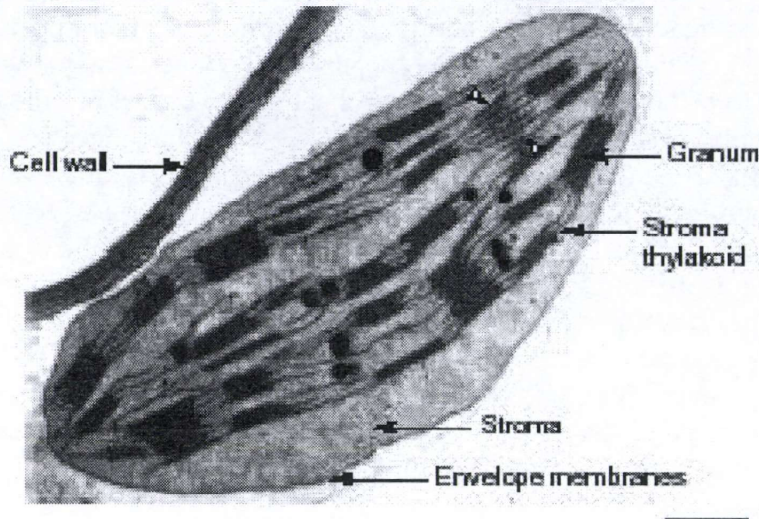
In a more frequently used, dangerously attractive model the grana are interconnected by tubes, which form a fret (Fig. 2b). This model is based on electron micrographs that suffer from a serious fixation artifact. Although tubular frets would in principle be consistent with most functions, they simply do not exist. Hence, this model should not be used, even for purposes of illustration, it is still found in many textbooks.

The folded membrane model (Fig. 2c) is most commonly used in research papers as a close-up view of the granum-stroma connection. This was

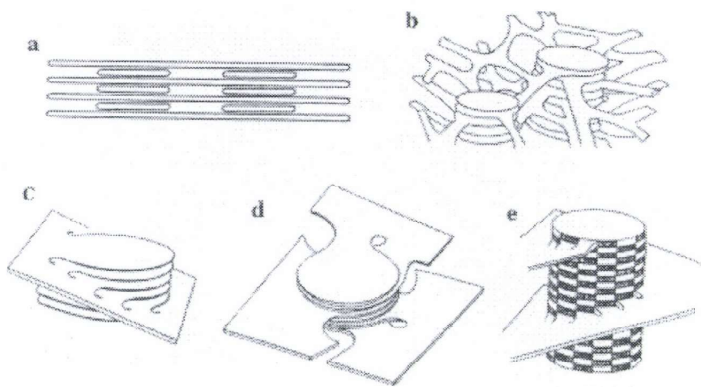
originally proposed for the illustration of the lateral heterogeneity. In this model, the stacking is thought to originate from invaginations. According to the model, stroma thylakoids do not directly continue the granum thylakoids. Instead, they form a 'fork': their 'upper' and 'lower' membranes are considered to originate from a layer 'above' and a layer 'below' the stacked pair of membranes. Such an arrangement, i.e. the interconnection of two granum compartments by a stroma thylakoid, can in fact, albeit rarely, be seen in electron micrographs. The reason for the appearance of 'forks' will be clear from the model based on serial sections. The main problem with this model is that, as ultrastructural studies on developing chloroplasts have revealed, the initiation of grana can be accounted for by the formation of multiple perforations, and by the overlapping of thylakoids at these slits, rather than invagination. It is still the model that is most frequently used in reviews and research papers; also available in a more elaborated form as a 3D computer model (Arvidsson and Sundby 1999).

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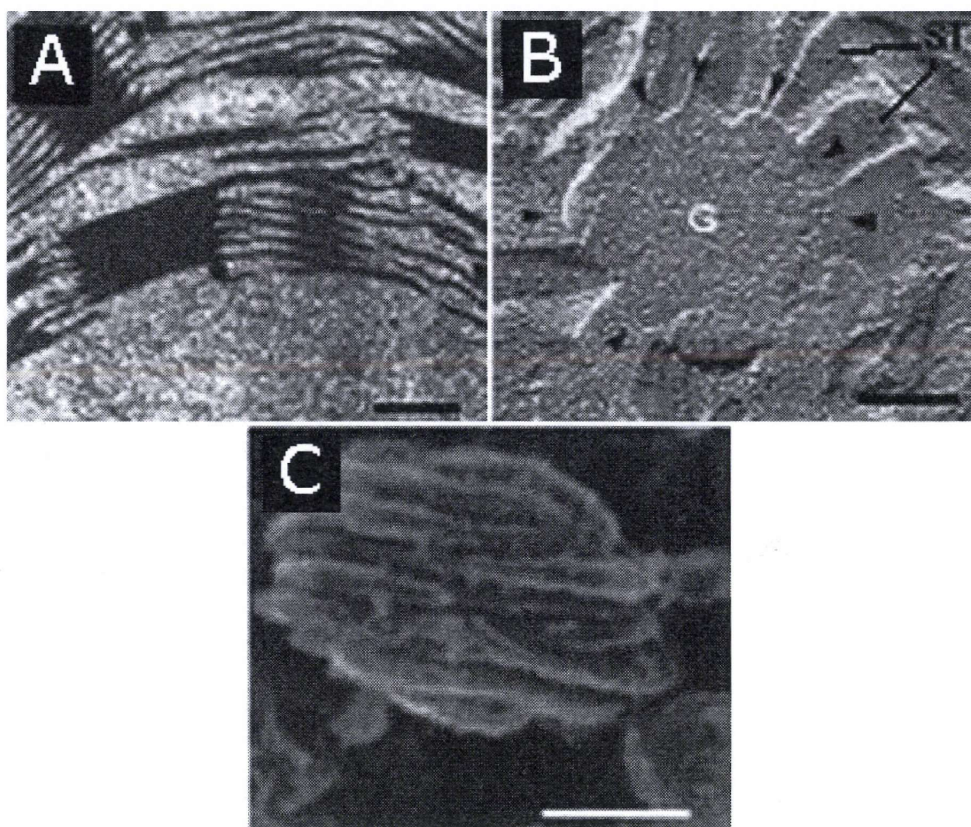


**Fig. 1** Electron micrograph of a higher plant chloroplast.

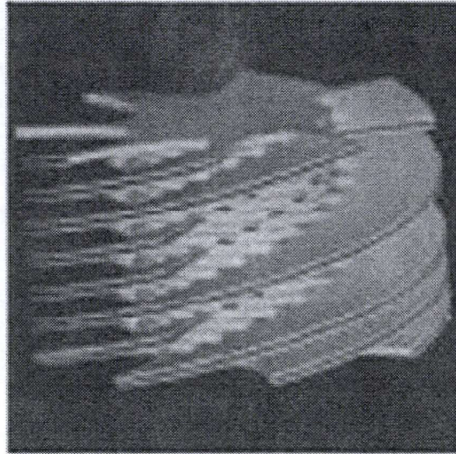


**Fig. 2** Development of models on higher plant chloroplast lamellar arrangement. (Redrawn after different authors) (a) First model proposed by Menke (1960) showing every second granum lamellae continues into the stroma and extends through several grana; (b) Cylindrical grana are interconnected by an anastomosing system of lamellar channels (Weier et al., 1963); (c) Stroma lamella intersect the granum compartments at an angle connecting them together (Heslop-Harrison, 1963); (d) Single stroma lamella interconnect the granum compartments in a helical manner (Wehrmeyer, 1964); (e) Continuous, large helices of stroma lamellae around the granum (Paolillo, 1970).

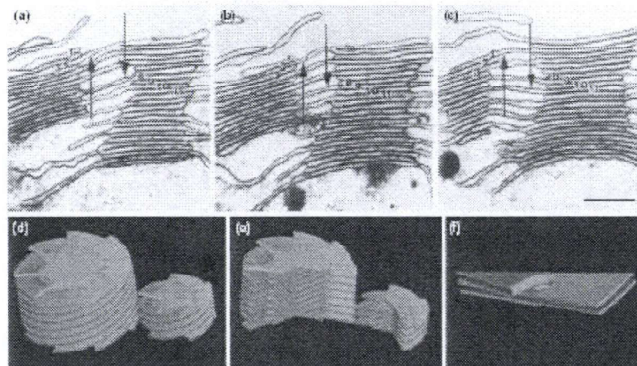




**Fig. 3** Evidences of helical arrangement of stroma membranes around the granum by different electron microscopic techniques. (A) Part of a 'conventional' thin section of a chloroplast. On the right hand side, the micrograph shows a granum-stroma assembly with a near-median section, i.e. cut close to the margin; hence, virtually only the stroma thylakoids are to be seen, tilted at an angle of approx.  $20^\circ$  with respect to the main axis of the granum. (B) On freeze-fractured chloroplast when the fractured plane is parallel with the granum thylakoid (G), stroma thylakoid junctions (arrowheads) are arranged in a circular fashion that surrounds the granum membrane. (C) Scanning electron micrograph of an isolated granum; after sonication, the stroma thylakoids are broken down from the more rigid granum body; the narrow strips of remaining stroma lamellae exhibit a helical arrangement. (bars  $0.2\ \mu\text{m}$ .)

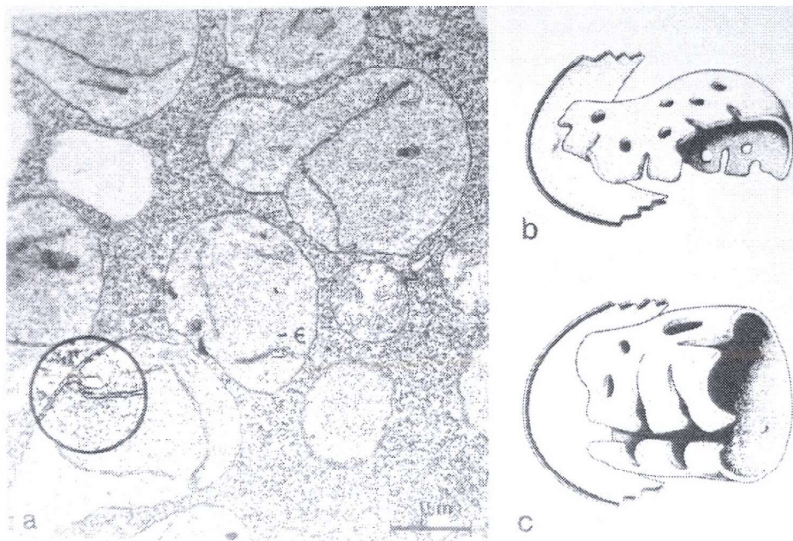


**Fig. 4** 'Computerized form' of the 3D model constructed from electron micrographs of full serial sectioning of a granum-stroma assembly. In the model, some stroma thylakoids are removed from the granum stacks to show the slits where the two types of thylakoids are connected.

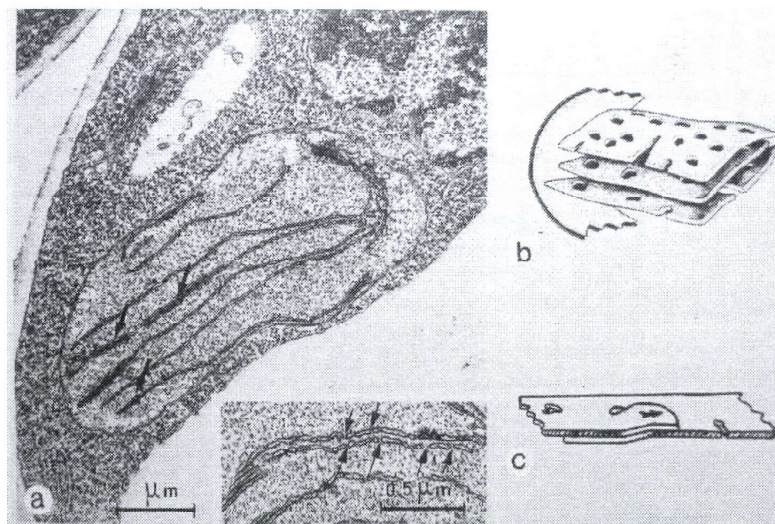


**Fig. 5** Thin sections from a full series (a-c), and different views of the 3D computer model of adjacent granum-stroma assemblies (d-e). It can be seen that the stroma thylakoids 'continue' the granum lamellae. However, for their helical arrangement the stroma thylakoids in different sections do not always run to the same thylakoid of the granum (indicated by the arrows). It is also clear (a-c) that there is a significant variation in the diameter of the stacks; this is not reflected in the schematic views in (d) and (e) which merely illustrate the fusion at the outer edges of the stroma membranes of the two grana, i.e. of the two granum-stroma units. (f) The key structural element, the 'unit cell' of the mathematical model of the granum-stroma assembly.

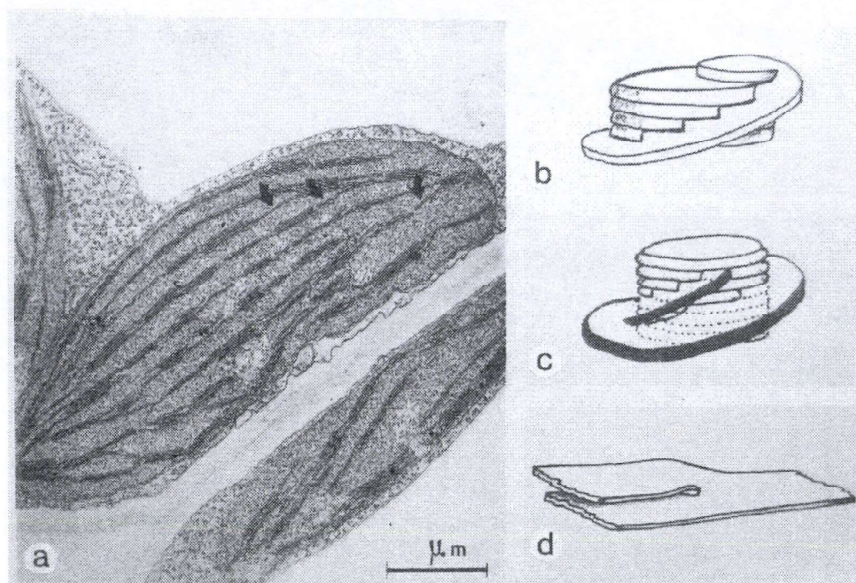




**Fig. 6** *Lolium* proplastids from meristematic tissue (a) and two reconstituted 3-dimensional views of lamellae (b and c). Circular inset shows invagination of inner plastid envelope.



**Fig. 7** Plastid profile from early stage of differentiation (a) showing parallel lamellar array with lamellar interruptions (inset-arrows) and juvenile grana stacks (arrows). At the right end of the plastid lamellar connections are evident as „parent entity” (arrow-heads). In spatial view these lamellae are independent through several sections (b) and the lamellar interruptions are revealed as slits or holes. (c) 3-dimensional reconstitution of a juvenile granum.



**Fig. 8** *Plastid profile from young chloroplast (a) showing fret connections between parent lamellar sheets (arrows). Three-dimensional reconstitutions of developing granum showing new compartment formation via overtopping (b), additional helix formation via splitting of primary helix (c) and thylakoid splitting on the lamellar sheet (d).*

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